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Research Articles

Chimeric α_2-,β_2-Adrenergic Receptors: Delineation of Domains Involved in Effector Coupling and Ligand Binding Specificity

BRIAN K. KOBILKA, TONG SUN KOBILKA, KIEFER DANIEL, JOHN W. REGAN, MARC G. CARON, ROBERT J. LEFKOWITZ

The α_2 and β_2 adrenergic receptors, both of which are activated by epinephrine, but which can be differentiated by selective drugs, have opposite effects (inhibitory and stimulatory) on the adenylyl cyclase system. The two receptors are homologous with each other, rhodopsin, and other receptors coupled to guanine nucleotide regulatory proteins and they contain seven hydrophobic domains, which may represent transmembrane spanning segments. The function of specific structural domains of these receptors was determined after construction and expression of a series of chimeric α_2-,β_2-adrenergic receptor genes. The specificity for coupling to the stimulatory guanine nucleotide regulatory protein lies within a region extending from the amino terminus of the fifth hydrophobic domain to the carboxyl terminus of the sixth. Major determinants of α_2- and β_2-adrenergic receptor agonist and antagonist ligand binding specificity are contained within the seventh membrane spanning domain. Chimeric receptors should prove useful for elucidating the structural basis of receptor function.

THE ADRENERGIC RECEPTORS (α_1-, α_2-, β_1-, AND β_2-), which mediate the physiological effects of catecholamines, belong to the family of plasma membrane receptors that are coupled to guanine nucleotide regulatory proteins (G proteins) (*1*). This receptor family also includes rhodopsin and the visual color opsins, the muscarinic cholinergic receptors, and many other neurotransmitter receptors and receptors for peptide hormones. A common feature of G protein–coupled receptors is that agonist occupancy of the receptor leads to receptor activation of a G protein, which in turn modulates the activity of an effector enzyme or ion channel. Several of the G protein–coupled receptors (including the major subtypes of adrenergic receptors) have been cloned and found to share structural features with rhodopsin (*2*). The most consistently conserved of these features is the existence of seven clusters of hydrophobic amino acids. In addition, there is significant amino acid sequence similarity among these receptors, which is most striking in the hydrophobic domains. For bovine rhodopsin, physical and biochemical studies have revealed that these hydrophobic domains may form seven alpha helices that span the lipid bilayer (*3*). It has been suggested that these alpha helices form a pocket for the chromophore 11-*cis*-retinal (*3*). Thus, in an analogous fashion, the hydrophobic domains of the adrenergic receptors may form a pocket in the plasma membrane for binding ligands.

Because so many different hormones, neurotransmitters, and drug receptors are likely to have structures homologous with the adrenergic receptors, it is necessary to achieve an understanding of the structural basis for the various functional properties of these receptors, in particular the specificity of ligand binding and effector coupling. This has been done heretofore (i) by mutagenesis, especially the deletion of specific peptide sequences (*4–6*), and (ii) biochemically, where proteases have been used to cleave defined peptide segments from the digitonin solubilized receptor (*7*). These methods, although useful in delineating regions of the receptor that do not influence its function, suffer from difficulties in that it is difficult to draw compelling inferences about the role of specific domains based on loss of functions.

In order to circumvent such problems, and to establish a potentially general approach to the study of G protein–coupled receptors so that positive inferences can be drawn about functions associated with specific receptor domains, we have constructed and expressed a series of chimeric α_2,β_2-adrenergic receptor genes. All of the subtypes of adrenergic receptors are activated by epinephrine, but they differ in their affinity for various subtype selective agonists and antagonists. Furthermore, the β_2-adrenergic receptors (β_2-AR's) couple to G_s (the stimulatory G protein for adenylyl cyclase) while the α_2-adrenergic receptors (α_2-AR's) couple to G_i (the inhibitory G protein for adenylyl cyclase). These two receptors therefore, respectively, stimulate and inhibit the enzyme. By studying the ligand binding and adenylyl cyclase activating properties of these chimeric receptors, in which various regions of the α_2- and β_2-adrenergic receptors have been interchanged, we have deduced structural domains that determine the specificity of ligand binding and effector coupling.

The α_2- and β_2-adrenergic receptors. We have described the cloning of the genes for both the human α_2-AR (*8*) and the human β_2-AR (*9*). Both genes have been expressed in *Xenopus laevis* oocytes by injecting the oocytes with receptor-specific mRNA (*8, 10*). Receptors expressed in this way can be detected by binding to specific radioactively labeled ligands. [^{125}I]Cyanopindolol can be used to detect expressed β_2-AR (*10*). The β_2-AR expressed in *Xenopus* oocyte membranes has an affinity for [^{125}I]cyanopindolol of 63 p*M* and has a typical β_2-AR agonist order of potency, with

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isoproterenol (β-AR agonist) being more potent than epinephrine (α_2- and β-AR agonist), which in turn is much more potent than *p*-aminoclonidine (α_2-AR agonist) (Table 1). These agonists, with the exception of *p*-aminoclonidine, stimulate β_2-AR's, expressed in *Xenopus* oocyte membranes, to activate endogeneous adenylyl cyclase (Table 2).

In contrast to the β_2-AR, α_2-AR expressed in *Xenopus* oocytes cannot be detected with $[^{125}I]$cyanopindolol, but instead binds $[^3H]$yohimbine (α_2-AR antagonist) with high affinity (2.5 n*M*). Competition binding studies with $[^3H]$yohimbine for α_2-AR expressed in *Xenopus* oocytes show a typical α_2-AR agonist order of potency, with *p*-aminoclonidine (α_2-AR agonist) being more potent than epinephrine (α_2- and β-AR agonist), which is much more potent than isoproterenol (β-AR agonist). These binding studies on α_2-AR expressed in *Xenopus* oocytes (*8*) are in agreement with studies on α_2-AR expressed in simian COS-7 cells (Table 3). Thus, like the β_2-AR, expression of the α_2-AR in *Xenopus* oocyte membranes can be documented and characterized by ligand binding. However, unlike the β_2-AR, a functional interaction of adenylyl cyclase with the α_2-AR expressed in *Xenopus* oocyte membranes has not been observed. Thus, stimulation of α_2-AR in *Xenopus* oocyte membranes does not lead to inhibition of adenylyl cyclase activity.

Chimeric receptors. To determine which structural domains of these two receptors confer specificity for agonist and antagonist binding as well as G protein coupling, we constructed ten chimeric receptor genes from the human β_2-AR and human platelet α_2-AR genes. These chimeric receptor genes were expressed in *Xenopus* oocytes and COS-7 cells, and the ability of the chimeric receptors to bind β_2-AR– and α_2-AR–specific ligands and to activate adenylyl cyclase was determined. When the ligand binding properties and G protein–coupling specificities of the various chimeric receptors are correlated with the α_2-AR and β_2-AR amino acid sequences of these chimeric receptors, it is possible to assign functional properties to specific structural domains.

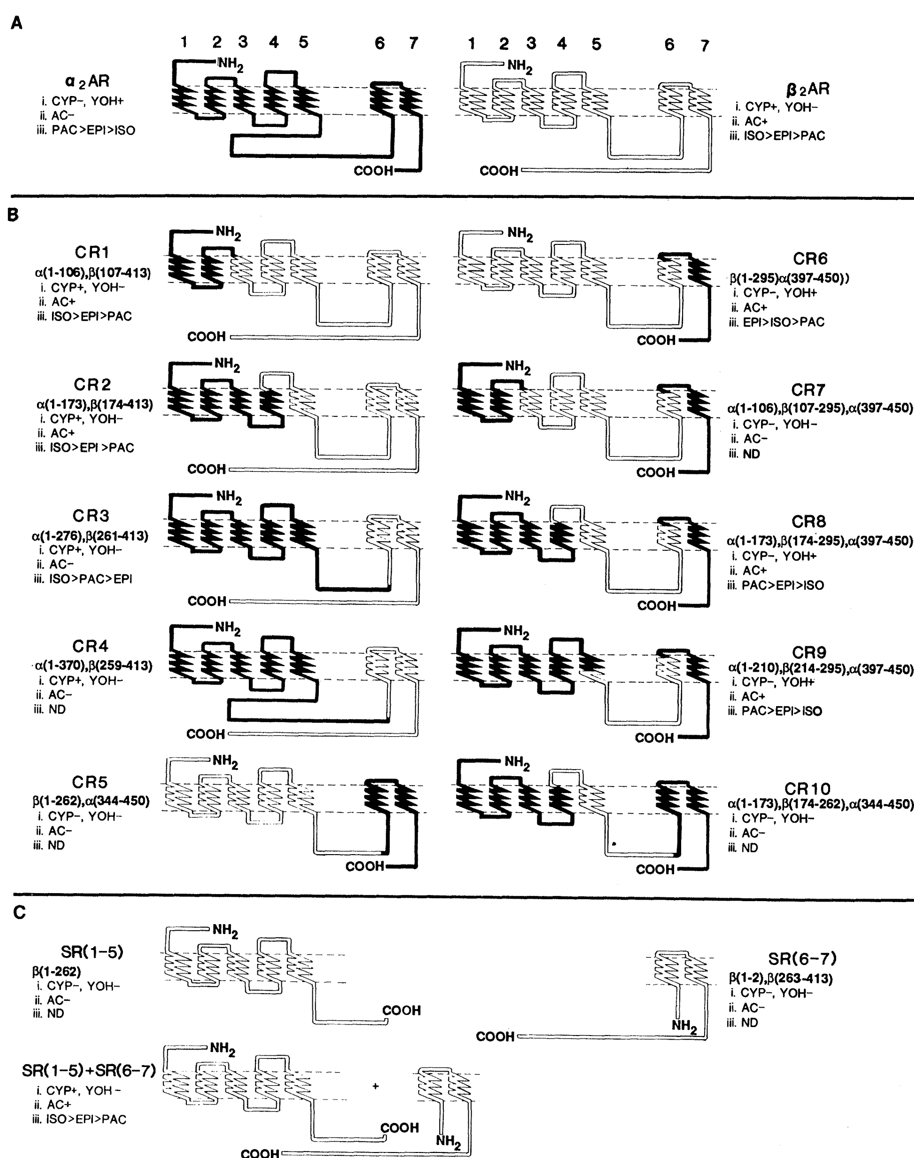

Fig. 1. (**A**) Diagram of the wild-type, α_2-adrenergic receptor (α_2-AR) and the wild-type, β_2 receptor (β_2-AR). The hydrophobic domains are shown as forming α-helices that span the plasma membrane. These putative α helices are numbered 1 to 7 from NH_2-terminus (extracellular) to the COOH-terminus (intracellular). (**B**) Chimeric receptors made from combinations of the wild-type α_2-AR and β_2-AR. The α_2-AR sequence is indicated by a solid line and β_2-AR sequence is indicated by an open line. The α_2-AR (α) and β_2-AR (β) amino acid sequences from NH_2- to COOH-terminus are indicated in parentheses beside each chimeric receptor. (**C**) Split receptors. SR(1–5) represents a truncation of the β_2-AR after amino acid 262 while SR(*6–7*) represents the β_2-AR in which amino acids 3 to 261 have been deleted. Beside each receptor is a summary of the functional characteristics of the receptor expressed in *Xenopus laevis* oocytes or COS-7 cells (or both). The functional properties include: (i) the ability to bind the β_2-AR antagonist ^{125}I-labeled cyanopindolol (CYP) and the α_2-AR–specific antagonist $[^3H]$yohimbine (YOH); (ii) the ability to couple to G_s and activate adenylyl cyclase (AC) after stimulation by epinephrine; and (iii) the relative potency of the α_2- and β_2-AR agonist epinephrine (EPI), the α_2-AR receptor–specific agonist *p*-aminoclonidine (PAC), and the β-AR–specific agonist isoproterenol (ISO) for the receptor as determined by ligand binding studies or adenylyl cyclase activation (or both). (ND, not determined.) Chimeric and split receptor genes were constructed by splicing desired restriction endonuclease fragments from the wild-type receptor genes with synthetic oligonucleotide adapters. The restriction endonuclease fragments encoding the desired structural domains of the α_2-AR and β_2-AR were isolated by preparative agarose gel electrophoresis. DNA sequences encoding amino acids not encoded by the DNA in these fragments were synthesized (Applied Biosystems model 380 B DNA synthesizer), so that the restriction fragments from the wild-type receptors plus the synthetic oligonucleotide adapters together contain all sequences necessary to encode a complete chimeric receptor.The oligonucleotides were phosphorylated at the 5′ hydroxyl and annealed before the ligation reaction.
The recombinant genes were identified by restriction endonuclease mapping, and the splice junctions were evaluated by dideoxy sequencing with the use of a denatured double-stranded DNA template (*14*). To ensure uniformity in the expression of the chimeric receptor, split receptors, and wild-type receptors, the 3′ and 5′ untranslated regions of all genes were derived from the β_2-AR cDNA (*9*). Receptor genes were expressed in *Xenopus laevis* oocytes by injecting oocytes with mRNA transcribed from the receptor gene ligated into pSP65 as described for the α_2-AR and β_2-AR (*8*, *10*). Expression of the genes in COS-7 cells was done by transfecting cells with genes cloned into pBC12MI in the presence of DEAE-dextran (*15*). Adenylyl cyclase and ligand binding assays are described below.

The structures of each of the ten chimeric receptors and the ability of each chimeric receptor to bind to [^{125}I]cyanopindolol or [^{3}H]yohimbine and to activate adenylyl cyclase after stimulation with epinephrine are compared in Fig. 1B. Chimeric receptors (CR) 1, 2, 3, and 4, expressed in *Xenopus* oocytes, were able to bind [^{125}I]cyanopindolol. Since CR 3 and CR 4 are structurally similar with respect to the composition of their putative membrane spanning domains, detailed pharmacologic studies were done on CR 3 as well as CR 1 and CR 2. Saturation binding isotherms and competition binding studies were done on the β_2-AR and on CR 1, CR 2, and CR 3 to determine the affinity constants for the β-AR antagonists [^{125}I]cyanopindolol and alprenolol and the agonists isoproterenol, epinephrine, and *p*-aminoclonidine (Fig. 2 and Table 1). The α_2-AR antagonist yohimbine at a concentration of 0.1 m*M* did not compete with [^{125}I]cyanopindolol for binding sites on these chimeric receptors.

Table 1. Iodine-125–labeled cyanopindolol binding studies on the human β_2-AR and chimeric receptors (CR). Ligand binding studies were performed as described in Fig. 2. Saturation isotherms were performed by incubating membranes with varying concentrations of ^{125}I-labeled cyanopindolol in the presence or absence of $10^{-5}M$ alprenolol. Equilibrium dissociation constants were determined from saturation isotherms (K_d) and competition binding experiments (K_i). Saturation isotherm data were analyzed by a nonlinear least-squares curve-fitting technique (*16*). Competition curves were analyzed according to a four-parameter logistic equation to determine EC_{50} values (*17*). The K_d values represent means of two independent experiments each performed in duplicate. The two independent pK_d values were within 10 percent of the mean pK_d. The K_i values are from a single experiment performed in duplicate (Fig. 2) in which all receptors were studied with the same stock solutions of competing ligands. These values are representative of two other independent experiments for each receptor in which the pK_i values were within 10 percent.

Receptor	Equilibrium dissociation constants				
	Antagonists		Agonists		
	CYP K_d (p*M*)	ALP K_i (n*M*)	ISO K_i (μ*M*)	EPI K_i (μ*M*)	PAC K_i (μ*M*)
β_2-AR	63	1.2	0.42	2.9	770
CR 1	18	2.0	5.4	70	510
CR 2	15	24	16	45	150
CR 3	57	2.0	180	1000	390

Table 2. Agonist mediated stimulation of adenylyl cyclase by the β_2-AR, the α_2-AR, and chimeric receptors expressed in *Xenopus laevis* oocyte membranes.

Receptor	Stimulation of adenylyl cyclase* (%)	EC_{50} for agonist stimulation (μ*M*)†		
		ISO	EPI	PAC
β_2-AR	100	0.21	0.41	NS‡
α_2-AR	0	NS	NS	NS
CR 1	56	2.2	27	NS
CR 2	21	16	60	NS
CR 6	21	280	110	320
CR 8	40	79	39	1.8
CR 9	35	NS	560	40

*The maximal adenylyl cyclase activity stimulated by a chimeric receptor in the presence of $10^{-3}M$ epinephrine expressed as a percentage of the maximal adenylyl cyclase stimulated by the β_2-AR. These values represent the mean of two independent experiments done in duplicate, one of which is shown in Fig. 4. †The concentration of an agonist that produces one half the maximal stimulation of adenylyl cyclase activity evoked by that agonist at 1 m*M*. The EC_{50} values represent the mean of two to three independent experiments done in duplicate (Fig. 5) in which the individual pIC_{50} (negative logarithm of the median inhibition concentration) values were within 10 percent of the mean. Agonists include epinephrine (EPI), isoproterenol (ISO), and *p*-aminoclonidine (PAC). Most of the dose response curves for the chimeric receptors do not plateau by $10^{-3}M$ agonist and therefore the EC_{50} values may be artificially low; however, they are nonetheless useful in comparing the relative potency of agonists for a given receptor. ‡No stimulation of adenylyl cyclase above basal.

[^{3}H]Yohimbine (α_2-AR antagonist) binding was assayed in COS-7 cell membranes after transient transfection of these cells with the chimeric receptor genes. While CR 6 bound [^{3}H]yohimbine weakly, only CR 8 (Fig. 3) and CR 9 bound [^{3}H]yohimbine with an affinity high enough to permit determination of affinity constants for agonists and antagonists (Table 3).

The ability of each chimeric receptor to couple to G_s and thus activate adenylyl cyclase was determined by studying epinephrine-stimulated adenylyl cyclase activity in oocyte membranes expressing the chimeric receptor. Control oocyte membranes exhibited little or no epinephrine-stimulated adenylyl cyclase activity. Epinephrine was used because it is an agonist for both α_2-AR and β_2-AR, and thus would be expected to act as an agonist for an α_2-β_2-chimeric receptor. Chimeric receptors 1, 2, 6, 8, and 9 were capable of activating adenylyl cyclase while CR's 3, 4, 5, 7, and 10 were not. An epinephrine dose response study was done to determine the efficiency of agonist stimulated receptor activation of adenylyl cyclase for CR's 1, 2, 6, 8, and 9 relative to the β_2-AR (Fig. 4 and Table 2). The agonist potency for adenylyl cyclase stimulation of each chimeric receptor was determined from results of isoproterenol, epinephrine, and *p*-aminoclonidine dose response studies on each chimeric receptor capable of mediating epinephrine-stimulatable adenylyl cyclase activity (Fig. 5 and Table 2).

G protein coupling. One of our goals was to locate the region of the β_2-AR that is responsible for coupling to G_s and to determine whether this domain is also involved in ligand binding. Of the chimeric receptors that can activate adenylyl cyclase, CR 8 and CR 9 contain the shortest stretches of β_2-AR. Furthermore, the agonist order of potency for both cyclase activation and ligand binding for both of these chimeric receptors resembles α_2-AR in that *p*-aminoclonidine > epinephrine > isoproterenol (Figs. 3 and 5 and Tables 2 and 3). The β_2-AR sequence in CR 8 extends from amino acid 174 at the NH_2-terminal portion of the second putative extracytoplasmic loop, through the fifth hydrophobic domain and the third cytoplasmic loop, and ending at amino acid 295 at the COOH-terminal portion of the sixth hydrophobic domain (Fig. 6). CR 10, which contains β_2-AR amino acid sequence 174 to 261 does not activate adenylyl cyclase. Chimeric receptor 3, which contains β_2-AR amino acid sequence 262 to 413 also does not activate adenylyl cyclase even though it binds [^{125}I]cyanopindolol. Chimeric receptor 9 contains β_2-AR amino acid residues 215 to 295 and activates adenylyl cyclase, but the efficiency of this activation is weak compared to activation by CR 8, as can be seen by comparing the

Table 3. [^{3}H]Yohimbine binding studies with the human α_2-AR and chimeric receptors (CRs). Ligand binding studies were performed as described in Fig. 3. Saturation isotherms were performed by incubating membranes with varying concentrations of [^{3}H]yohimbine in the presence or absence of $10^{-5}M$ unlabeled yohimbine. Data were obtained and analyzed as described in the legend to Table 1. The K_d values represent means of two independent experiments each performed in duplicate. The two independent pK_d values were within 10 percent of the mean pK_d. The K_i values are from a single experiment in which all receptors were studied with the same stock solutions of competing ligands. These values are representative of two other independent experiments from each receptor in which the pK_i values were within 10 percent.

Receptor	Equilibrium dissociation constants				
	Antagonists		Agonists, K_i (μ*M*)		
	YOH K_d (n*M*)	ALP K_i (μ*M*)	ISO	EPI	PAC
α_2-AR	2.6	5.4	230	1.0	0.074
CR 8	49	3.5	590	40	1.3
CR 9	49	5.5	>1000	46	0.72

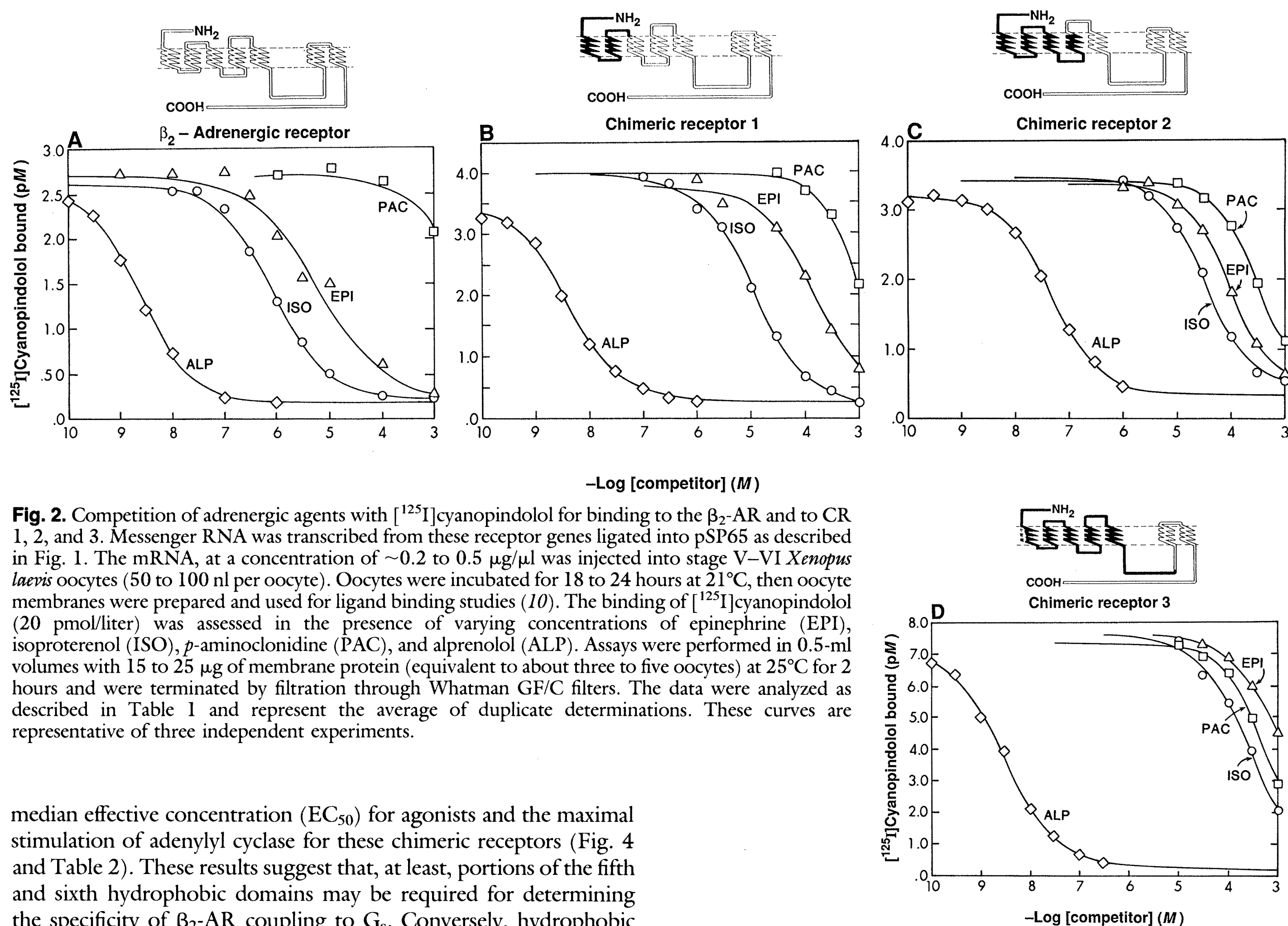

Fig. 2. Competition of adrenergic agents with [^{125}I]cyanopindolol for binding to the β_2-AR and to CR 1, 2, and 3. Messenger RNA was transcribed from these receptor genes ligated into pSP65 as described in Fig. 1. The mRNA, at a concentration of ~0.2 to 0.5 μg/μl was injected into stage V–VI *Xenopus laevis* oocytes (50 to 100 nl per oocyte). Oocytes were incubated for 18 to 24 hours at 21°C, then oocyte membranes were prepared and used for ligand binding studies (*10*). The binding of [^{125}I]cyanopindolol (20 pmol/liter) was assessed in the presence of varying concentrations of epinephrine (EPI), isoproterenol (ISO), *p*-aminoclonidine (PAC), and alprenolol (ALP). Assays were performed in 0.5-ml volumes with 15 to 25 μg of membrane protein (equivalent to about three to five oocytes) at 25°C for 2 hours and were terminated by filtration through Whatman GF/C filters. The data were analyzed as described in Table 1 and represent the average of duplicate determinations. These curves are representative of three independent experiments.

median effective concentration (EC_{50}) for agonists and the maximal stimulation of adenylyl cyclase for these chimeric receptors (Fig. 4 and Table 2). These results suggest that, at least, portions of the fifth and sixth hydrophobic domains may be required for determining the specificity of β_2-AR coupling to G_s. Conversely, hydrophobic domains 1, 2, 3, 4, and 7 as well as the first and second cytoplasmic loops and the COOH-terminus appear to have little influence in determining the specificity for G protein coupling.

Studies on site-directed mutagenesis of the hamster β_2-AR (*5, 6*) and proteolysis of digitonin solubilized turkey β-AR (*7*) have addressed the issue of which structural domains may be involved in coupling of the β-AR to G_s. Deletion of several small segments of the third cytoplasmic loop of the hamster β_2-AR does not affect G protein coupling (*5, 6*). The region of the human β_2-AR analogous to the hamster β_2-AR in the region of these deletions extends from amino acid residues 229 through 262 (Fig. 6). Also, deletion of sequences at the NH_2- and COOH-terminal portions of the third cytoplasmic loop in the hamster β_2-AR leads to loss of G_s activation (*6*). In the human β_2-AR (Fig. 6), these deletions would correspond to amino acid 222 to 229 and amino acid 258 to 270, respectively. These studies therefore provide clues to the potential sites of interaction between the β_2-AR and G_s; however, it is also possible that the negative effect of these deletion mutations might be due to an allosteric rather than a direct effect on the actual G protein coupling domain.

Proteolysis studies on the turkey β-AR suggest that deletion of even larger regions of the third cytoplasmic loop, and possibly of the fifth hydrophobic domain, do not affect the ability of the receptor to couple to G_s (*7*). However, with this approach it was difficult to define the precise position of some of the proteolytic cleavage sites.

Our results define a limited region of the human β_2-AR which, when placed in the analogous position of the human α_2-AR, confers the ability to couple to and activate G_s with an α_2-AR agonist order of potency. More detailed resolution of the precise sequences necessary for receptor-G_s coupling will be achieved by insertion of smaller segments of the β_2-AR into the α_2-AR and by single amino acid substitutions.

Ligand binding. A number of studies have suggested that the hydrophobic domains of the β_2-AR are involved in the formation of the ligand binding pocket. In constructing and studying the series of α_2- and β_2-AR chimeric receptors an attempt was made to determine which domains conferred ligand binding specificity for agonists and antagonists. Since each chimeric receptor is an artificial combination of α_2- and β_2-AR, it might not be expected to function as well as either of the native receptors (see below). Attention was therefore focused on the relative order of potencies for agonists and antagonists rather than the absolute affinities for the different agents. Thus, each chimeric receptor can be classified as having an α_2-AR or β_2-AR agonist or antagonist potency series. These determinations were made on the basis of both ligand binding (for those chimeric receptors capable of binding either [^{125}I]cyanopindolol or [^{3}H]yohimbine) and adenylyl cyclase assays.

A comparison of [^{125}I]cyanopindolol binding studies (Fig. 2 and Table 1) and adenylyl cyclase studies (Table 2) on the native β_2-AR with those on CR 1, 2, and 3 suggests that hydrophobic domains 1 to 5 are not involved in a major way in determining β_2-AR antagonist specificity. All of these chimeric receptors bind [^{125}I]cyanopindolol with an affinity equivalent to or higher than the native β_2-AR (Table 1).

A somewhat different picture emerges for the agonists. The affinity of all agonists for CR 1, 2, and 3 was significantly lower than for the β_2-AR. Moreover, a progressively changing specificity for

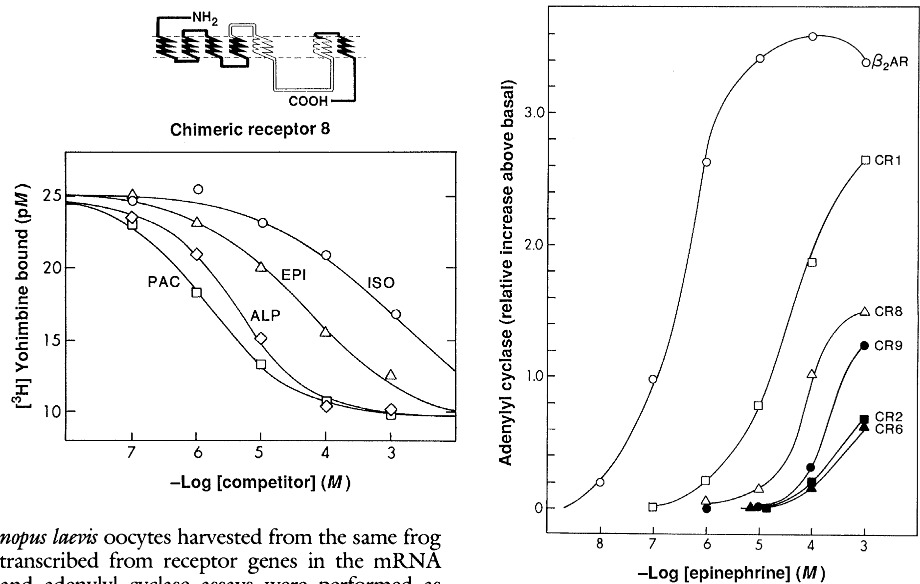

Fig. 3 (left). Competition of adrenergic agents with [^{3}H]yohimbine for binding to CR 8 expressed in COS-7 cells. Cells were rinsed with phosphate-buffered saline, scraped from the culture flasks, and homogenized in ice-cold 5 m*M* tris-HCl, *p*H 7.4, and 2 m*M* EDTA with a Polytron (five bursts of 5 seconds at maximum setting). The lysate was adjusted to 75 m*M* tris-HCl, *p*H 7.4, 12.5 m*M* $MgCl_2$ and 2 m*M* EDTA and used for ligand binding studies. The binding of [^{3}H]yohimbine (20 n*M*) was assessed in the presence of varying concentrations of the indicated agonists and antagonists. Assays were performed in 0.5-ml volumes with 50 to 100 μg of membrane protein for 2 hours at 25°C, and the reactions were terminated by filtration through Whatman GF/C glass fiber filters. Data were analyzed as described in Table 3 and represent the average of duplicate determinations. These curves are representative of three independent experiments. **Fig. 4 (right).** Dose response of epinephrine for stimulation of adenylyl cyclase activity mediated by the β_2-AR and chimeric receptors. *Xenopus laevis* oocytes harvested from the same frog were injected with equivalent amounts of mRNA transcribed from receptor genes in the mRNA expression vector pSP65. Membrane preparations and adenylyl cyclase assays were performed as described (*10*). Each data point represents the mean of triplicate determinations. Each determination was performed on membranes from 25 to 35 mRNA-injected *Xenopus laevis* oocytes. Adenylyl cyclase activity above basal (unstimulated) activity is expressed as a fraction of the basal activity, that is, the difference between the agonist stimulated value (X) and the basal value (Y) divided by the basal value [$(X - Y)/Y$]. Thus a value of 1.0 represents a doubling of the basal value by agonist or a 100 percent increase above basal. Typical basal values range from 0.3 to 1.0 pmol of cyclic AMP per milligram of membrane protein per minute. The experiment shown is representative of two independent experiments.

agonists can be appreciated by considering the ratio of K_i's for the α_2-AR agonist *p*-aminoclonidine and the β-AR agonist isoproterenol [K_i(PAC)/K_i(ISO)]. At the two extremes, the ratio of K_i(PAC) to K_i(ISO) was 1800 for the β_2-AR and 0.00032 for the α_2-AR. The values for the chimeric receptors were: CR 1, 94; CR 2, 9.4; and CR 3, 2.2. Thus, as the extent of α_2-AR sequence increases the receptor becomes progressively less "β_2-AR–like" in its agonist binding properties.

Our data on the chimeric receptors, in particular the ligand binding properties of CR 9, suggest that the sixth hydrophobic domain does not exert a major influence on either agonist or antagonist order of potency. However, the seventh hydrophobic domain appears to be a major determinant of both agonist and antagonist ligand binding specificity. A study of CR 6 shows that replacement of the seventh hydrophobic domain in the β_2-AR with the seventh hydrophobic domain from the α_2-AR leads to a loss of [^{125}I]cyanopindolol binding and the acquisition of the ability to bind [^{3}H]yohimbine, albeit with low affinity. Furthermore, isoproterenol is less potent than epinephrine in stimulating adenylyl cyclase via CR 6, and *p*-aminoclonidine is able to activate adenylyl cyclase (Table 2).

The importance of the seventh hydrophobic domain in conferring α_2-agonist and antagonist specificity can be further illustrated by comparing CR 2 and 8. In CR 2, hydrophobic domains 1 to 4 are derived from the α_2-AR and hydrophobic domains 5 to 7 are derived from the β_2-AR. This chimeric receptor exhibits predominantly β_2-AR ligand binding properties (Fig. 2C). Chimeric receptor 8 is made by changing the seventh hydrophobic domain in CR 2 from β_2-AR to α_2-AR (see Fig. 1B). In contrast to chimeric CR 2, CR 8 exhibits α_2-AR agonist and antagonist ligand binding properties (Fig. 3 and Table 3), and activates adenylyl cyclase with an α_2-AR agonist order of potency (Fig. 5). Thus, changing the seventh hydrophobic domain in CR 2 from β_2-AR to α_2-AR results in a change in the ratio of K_i(PAC) to K_i(ISO) from 9.4 to 0.0022.

These data indicate that most of the hydrophobic domains influence agonist ligand binding specificity, while antagonist ligand binding specificity (at least for [^{3}H]yohimbine, [^{125}I]cyanopindolol, and alprenolol) is influenced primarily by the seventh hydrophobic domain or the combination of the sixth and the seventh. Thus, CR 3, which contains only hydrophobic domains 6 and 7 from the β_2-AR, has affinities for [^{125}I]cyanopindolol and alprenolol that are close to the affinities of these ligands for the wild-type β_2-AR.

Split receptor. The role of hydrophobic domains 6 and 7 in binding to [^{125}I]cyanopindolol was then explored. The β_2-AR was expressed as two separate peptides (see Fig. 1C), one encoding amino acid 1 to 262, containing hydrophobic domains 1 to 5, SR(1–5), and the other containing hydrophobic domains 6 to 7, SR(6–7). We constructed SR(1–5) by inserting a termination codon after amino acid 262. This mutant does not bind ligands or activate adenylyl cyclase (*10*). We made SR(6–7) by deleting the region between the second amino acid of the β_2-AR and amino acid 262. It is possible to express SR(1–5) and SR(6–7) together in *Xenopus* oocytes and obtain a functional receptor with a K_d for [^{125}I]cyanopindolol of 44 p*M* and normal β_2-AR affinities for isoproterenol, epinephrine, and norepinephrine (Fig. 7). This "split receptor" can also activate adenylyl cyclase, although it is only ~25 percent as efficient as the wild-type β_2-AR in doing so. However, injection of mRNA for SR(6–7) alone does not lead to the expression of a ^{125}I-labeled cyanopindolol binding protein in the oocyte membranes. This suggests that, even though hydrophobic domain 7 (or 6 and 7) appear to be the major determinants of β_2-AR ligand binding specificity, this region of the molecule by itself is insufficient to bind β_2-AR ligands or activate adenylyl cylcase.

While these results suggest that the seventh hydrophobic domain is involved in dictating ligand binding specificity, it cannot be concluded that this hydrophobic domain forms the ligand binding pocket. This domain may confer ligand binding specificity by interaction with the domains directly involved in the formation of the binding site. The β-AR–specific photoaffinity antagonist pBABC covalently binds to a peptide in the second hydrophobic domain (*11*) suggesting that this domain may form or lie adjacent to the ligand binding pocket. Site-directed mutagenesis of various residues in different domains of β_2-AR leads to alteration of ligand binding properties (*4, 12*). While these findings may indicate that the mutated regions are involved in the formation of the ligand binding site, they may also be due to allosteric effects.

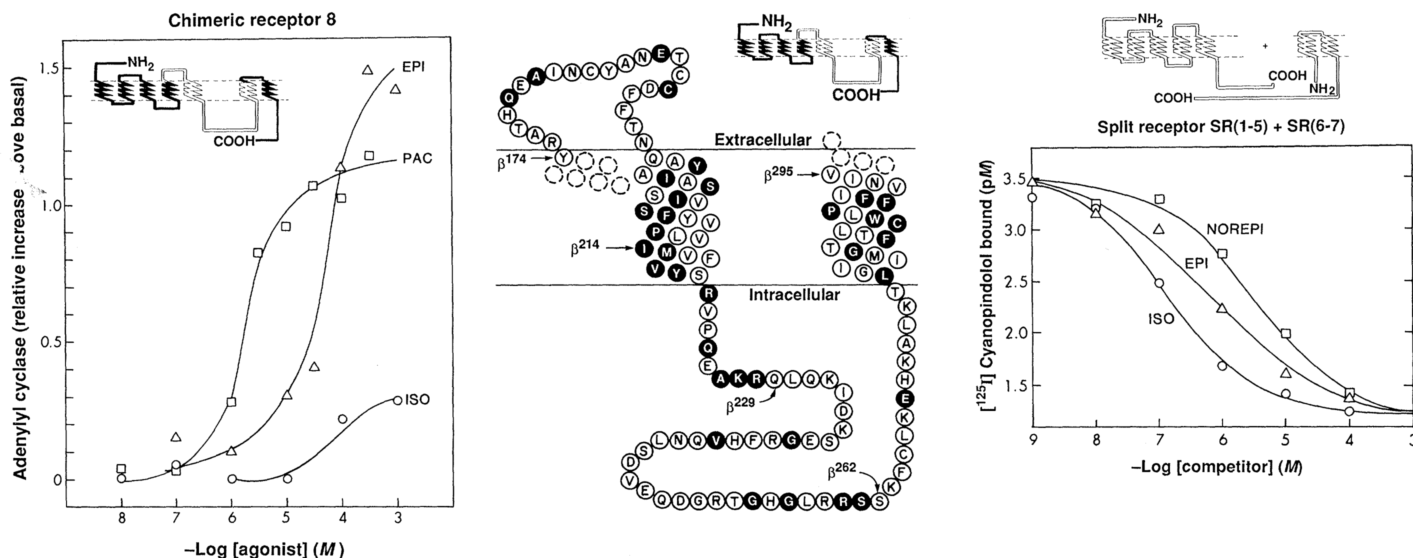

Fig. 5 (**left**). Adenylyl cyclase activity stimulated via CR 8. *Xenopus* oocyte membranes containing CR 8 were assayed for adenylyl cyclase at varying concentrations of the indicated agonists. The methods are described in Fig. 4. Each data point represents the mean of two independent experiments in which determinations were done in duplicate. Each determination was performed with membranes from 25 to 35 mRNA-injected *Xenopus laevis* oocytes. Adenylyl cyclase is expressed as described in Fig. 4. **Fig. 6** (**center**). β_2-AR amino acid sequence of CR 8. The diagram of CR 8 is shown at the top. The open line represents β_2-AR sequence. The amino acids derived from the β_2-AR are shown in the larger diagram as circles. Black circles with white letters represent amino acid identities between the α_2-AR and β_2-AR. The numbered amino acid residues are discussed in the text. **Fig. 7** (**right**). Agonist competition binding studies on split receptors. SR(1–5) and SR(6–7) were expressed together in *Xenopus laevis* oocytes. Messenger RNA transcribed from SR(1–5) and SR(6–7) genes in the mRNA expression vector pSP65 was mixed and injected into *Xenopus laevis* oocytes. After 24 hours, membranes were prepared and competition-binding was performed as described in Fig. 2. Varying concentrations of isoproterenol (ISO), epinephrine (EPI), and norepinephrine (NOREPI) competed for binding sites with 75 p*M* ^{125}I-labeled cyanopindolol. Data were analyzed as described in Table 1.

Possible arrangement of hydrophobic domains. The study of this set of chimeric receptors has provided insight into the function of various structural domains. However, these molecules may also provide clues about the arrangement of the various hydrophobic domains within the parent molecules. These hydrophobic domains may form α-helices that span the plasma membrane, as has been suggested by electron diffraction studies on bacteriorhodopsin (*13*). For the following discussion we therefore refer to the hydrophobic domains as membrane spanning α-helices. The arrangement of these α-helices with respect to each other might be dictated by the interactions of various charged, noncharged polar, and nonpolar amino acids as well as the possible formation of covalent bonds (that is, disulfide bridges). The less hydrophobic amino acids of these α helices are likely to project toward the interior of the molecule, while the more hydrophobic residues may form a boundary with the plasma membrane. The α helices that lie adjacent to each other have presumably evolved in such a way as to minimize steric and electrostatic repulsive forces between each other.

In the process of making chimeric receptors these favorable molecular interactions may be lost. For example, in making CR 1, α-helix 2 from the α_2-AR and α-helix 3 from the β_2-AR are forced to lie adjacent to each other. Furthermore, other α helices that may normally cluster around α-helices 1 and 2 from the β_2-AR may be less compatible with α-helices 1 and 2 from the α_2-AR. These molecular incompatibilities might be expected to destabilize the molecule, and thus make it less efficient or even nonfunctional. This is consistent with the finding that all chimeric receptors in this series are less efficient than either parent molecule in binding agonists, and less efficient than the β_2-AR in activating G_s. Furthermore, chimeric receptors containing two molecular splice junctions such as CR 7, 8, 9, and 10 (Fig. 1), might be expected to function even less well than chimeric receptors with only one splice junction. For example, while CR 1 and CR 6 are both able to couple to and activate G_s, CR 7, which contains both molecular splice junctions found in CR 1 and CR 6, is nonfunctional even though it contains the essential elements of β_2-AR necessary to activate G_s.

Chimeric receptor 8 is of particular interest, therefore, since it contains two molecular splice junctions, yet has a higher affinity for epinephrine (Tables 1 and 3) and is more efficient at activating adenylyl cyclase (Table 2) than either CR 2 or CR 6, each of which contains only one of the two molecular splice junctions found in CR 8 (Fig. 1). This observation might be explained by considering the possible arrangement of hydrophobic domains in the α_2-AR and various chimeric receptors (Fig. 8). The model proposes that α-helix 7 of the α_2-AR and CR 1, 6, 7, and 8 lie adjacent to α-helices 3 and 4. In CR 6 (Fig. 8B) α-helix 7 from the α_2-AR is paired with α-helix 3 and 4 from the β_2-AR and is therefore less stable. Similarly in CR 2 (Fig. 8C), α-helix 7 from the β_2-AR is paired with α-helices 3 and 4 from the α_2-AR. However, in CR 8 (Fig. 8D) α-helices 3, 4, and 7 are all from the α_2-AR. Thus, the potential molecular incompatibilities between α-helix 4 and α-helix 5 and between α-helix 6 and α-helix 7 in CR 8 are compensated for by the opportunity for α-helix 7 to interact normally with α-helices 3 and 4 from the same receptor. In the nonfunctional CR 7 (Fig. 8E), potential incompatibilities between α-helix 7 and α-helices 3 and 4 as well as between α-helix 2 and α-helix 3 and between α-helix 6 and α-helix 7 may contribute to the lack of activity of this receptor. Thus, on the basis of the functional capacity of chimeric receptors, it may be possible to predict the arrangement of α-helices within the parent molecules.

Only CR 5, 7, and 10 are nonfunctional; that is, they do not bind [^{3}H]yohimbine or [^{125}I]cyanopindolol, nor do they activate adenylyl cyclase. Sequence analysis of the splice junctions of these chimeric receptor genes confirmed that these chimeric receptors were properly constructed. Furthermore, in vitro translation of mRNA made from these chimeric receptor genes produced a protein of the predicted molecular size. While the lack of function of these chimeric

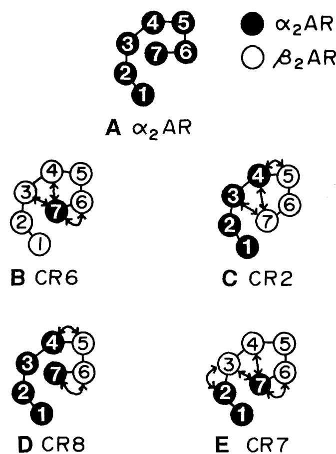

Fig. 8. Possible arrangement of hydrophobic domains for the α_2-AR and various chimeric receptors. The hydrophobic domains are depicted as α-helices as they would appear from above the plasma membrane (**A**). Alpha helices from the α_2-AR are shown as black circles with white numbers while those from β_2-AR are shown as white circles with black numbers. Potential destabilizing interactions between α-helices from different receptors are indicated by arrows. The model attempts to explain the observation that CR 8 (**D**) functions better than CR 6 (**B**), CR 2 (**C**), or CR 7 (**E**) as assessed by affinity for epinephrine (Tables 1 and 3) and by activation of adenylyl cyclase (Table 2).

receptors might be explained by molecular incompatibilities between α-helices as discussed above, it is also possible that these chimeric receptors failed to insert properly in the plasma membrane as a result of specific amino acid sequences created at the splice junctions. This may be particularly important for CR 5 and CR 10 which have a splice junction in the putative third cytoplasmic loop in a region where the α_2-AR and β_2-AR share little amino acid sequence similarity.

The results from our study of chimeric receptors made from the β_2-AR and α_2-AR have provided new insights into the functional role of several structural domains. The fifth and sixth hydrophobic domains and the third cytoplasmic loop are capable of conferring specificity for G_s coupling to the β_2-AR. The seventh hydrophobic domain of the α_2- and β_2-adrenergic receptors is a major determinant of both agonist and antagonist ligand binding specificity. Finally, several of the first five hydrophobic domains may contribute to agonist binding specificity. The strength of this approach for the study of these functionally complex molecules is that conclusions can be drawn from qualitative changes in receptor function, or from the acquisition of new functions that can be correlated with specific protein sequences. This is in contrast with more standard mutagenesis approaches where the end point is the loss of function resulting from amino acid deletions or substitutions. Our results provide an initial structural map for understanding the various functions of two model G protein–coupled receptors. The map requires further refinement and testing of its generality for understanding receptors of this class.

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